

Micellar Chromatography of Proteins

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Proteins are separated on a reversed-phase column with nonionic surfactant/phosphate buffer at neutral pH as mobile phase. Frontal experiments demonstrated the ethoxylated alcohol surfactant was sorbed to the column packing. Caution was required to assure that steady-state conditions were reached so that reproducible chromatography could be performed. As observed with widely used acidic mobile phases containing organic solvents, small changes in additive concentration effected large changes in retention, so that shallow gradients were needed for separations of protein mixtures. Retention of proteins is discussed in terms of van der Waals attraction and repulsion concepts.

Aqueous solutions containing sodium dodecyl sulfate at concentrations well above the critical micelle concentration have been proposed as selective mobile phases in reversed-phase liquid chromatography (1). Subsequent investigations (2) suggested that the behavior of substituted benzenes could be explained in such systems by consideration of a three-phase model: the distribution of solute between bulk water and the stationary phase, its distribution between the micelle and the stationary phase, and its distribution between bulk water and micelle. Plots of (stationary phase volume/adjusted retention volume) vs. the concentration of detergent in micelles were linear as predicted by the model. A recent study has demonstrated that the chromatographic efficiency of micelle containing systems could be increased by the addition of small quantities of alcohols to the mobile phase and raising the column temperature slightly. It was presumed that the stationary phase was not wetted in the absence of alcohol, resulting in slow mass transfer (3).

Concomitant with these developments, reversed-phase HPLC became an important tool in protein chemistry (4). Acidic mobile phases containing large proportions of alcohol or acetonitrile were employed to separate proteins. We have shown that no proteins are retained on the columns studied at alcohol concentrations between 40% and 65% (5). As the concentration of alcohol was reduced, a composition was reached below which protein could not be observed to elute. The capacity factor (k') increased from zero to a very large number over a very narrow range of mobile phase compositions. We showed further that protein retention in reversed-phase systems could be explained in terms of attractive or repulsive van der Waals interactions.

It would be advantageous to be able to apply the selectivity of reversed-phase chromatography to the separation and/or isolation of proteins nearer to physiological pH. Examination of sorption isotherms (6) revealed that alcoholic buffers did not desorb protein at pH 7. While buffers containing a poly(ethoxy alcohol) surfactant did not desorb protein at pH 2, they did desorb protein at pH 7 but with concentrations of surfactant apparently well above the critical micelle concentration. The purpose of the present investigation is to explore further the use of micellar mobile phases for the reversed-phase chromatography of proteins.

EXPERIMENTAL SECTION

Equipment. The high-performance liquid chromatograph was a Spectra-Physics Model 8000 B. External detectors used were fluorescence (Schoeffel Model 970), variable-wavelength UV (Schoeffel Model 770), and refractive index (Waters Model R 401). For frontal experiments, the mixing chambers and injector of the chromatograph were bypassed to reduce lag time and back mixing between the pump and the column. Surfactant was pumped from one reservoir for the specified time, then solvent was pumped from another reservoir until a return to the original base line was achieved. The column was 100-Å Supelcosil LC-8 (Supelco), 250 mm \times 4.6 mm i.d.

Chemicals. Mobile phases were prepared with ACS-grade reagents, HPLC quality water, and either an ethoxylated alcohol surfactant (Neodol 91-6), (Shell) or β -octylglucoside (Sigma) surfactant. Neodol 91-6 is a blend of C_9 , C_{10} , and C_{11} primary alcohols with an average of 6 mol of ethylene oxide per mole of alcohol. Sodium azide (0.02%) was added as bacteriostat.

Proteins were dissolved in the mobile phase (2 mg/mL), and filtered through 0.45- μ m bacteriological filters before chromatography. Myoglobin, prepared as described (7), was graciously supplied by J. B. Fox (ERRC). The other proteins were purchased as calibration standards for size-exclusion chromatography from Sigma, Pharmacia, or Polysciences.

Methods for measuring surface tensions were described previously (5).

RESULTS AND DISCUSSION

The commercial ethoxylated alcohol surfactant used in these studies contains molecules of several chain lengths and degrees of ethoxylation so that it has a critical micelle range rather than a point. This behavior is illustrated in Figure 1, where surface tension of the ethoxylated alcohol solution (LV) is seen to approach asymptotically a value of ca. 31 ergs/cm² with increasing surfactant concentration at pH 2.1. Similar behavior (not shown) was observed at pH 7 although the asymptote was a few ergs/cm² lower. The critical micelle concentration of octylglucoside solutions is about 20 times higher than that of the alcohol but has the same limiting surface tension (8). Since large quantities of this expensive compound are needed, its utility in chromatography is limited. Its potential was surveyed in this study, however, because it is used widely in protein isolations where small solution volumes are sufficient and because it is easily removed from protein isolates. Figure 1 also shows the surface tensions of bovine serum albumin and octadecane (simulated stationary phase) with increasing surfactant concentration, as determined with techniques described previously (5) and calculated from an equation of state approach (9). It has been proposed (10) that a necessary condition for the desorption of protein from a surface is that the surface tension of the solvent fall between that of the protein and the surface. These data, therefore, suggest that reversed-phase chromatography with this surfactant mobile phase at pH 2.1 would not be suitable for protein separations and, indeed, no elution of proteins was observed. Surface tensions of many proteins near conditions of physiological pH and ionic strength are known, however, to be in the range of 66–71 ergs/cm² (10). Therefore, it was expected that separations of proteins could be achieved with surfactant solutions at that pH.

Table I. Properties of Proteins of Low, Intermediate, and High Retention in Surfactant/Reversed Phase System (pH 7)

low retention				intermediate				high			
	mol wt ^a ($\times 10^3$)	pI ^c	H ^d ($\times 10^3$)		mol wt ^a ($\times 10^3$)	pI ^c	H ^d ($\times 10^3$)		mol wt ^a ($\times 10^3$)	pI ^c	H ^d ($\times 10^3$)
ovalbumin	45	4.6	1.11	bovine serum albumin	65	4.8	1.12	lysozyme	14	11.0	0.97
catalase	58 ^b	5.7		thyroglobulin	335 ^b	4.6		cytochrome c	13	10.0	1.11
carbonic anhydrase	32	7.3	1.06	chymotrypsinogen	23	9.2	1.05	β -lactoglobulin	18 ^b	5.2	1.23
ferritin	800	5.0		ribonuclease	14	9.6					
apoferritin	24 ^b		1.05								

^a Reference 11. ^b Subunit. ^c Reference 12. ^d Average hydrophobicity, ref 13.

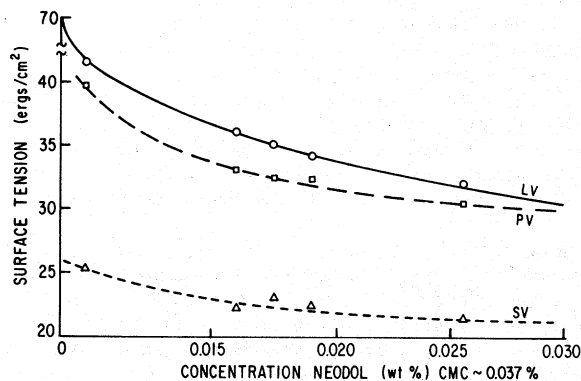


Figure 1. Surface tensions of alkane, bovine serum albumin, surfactant system: (LV) $R(OCH_2CH_2)_6OH/0.05$ M phosphate, pH 2.1; (PV) protein; (SV) alkane.

Initial experiments with various isocratic concentrations of surfactant as mobile phase followed by elution with alcohol/buffer mixtures at pH 2.1 to desorb protein not eluted with the former mobile phase produced inconsistent results. Batch equilibrations of surfactant containing buffer (pH 7) with reversed-phase packing material revealed that apparent concentrations well above the critical micelle range were required to wet the packing. To examine this interaction further, frontal chromatography with surfactant solution (pH 7) was performed. The trailing edge of a composite frontal profile for components of a mixture is the inverted mirror image of the leading edge. Since the frontalgram in Figure 2 does not demonstrate this behavior, it is interpreted as one produced by an interacting surfactant system. The first front, eluting near the system holdup volume (~ 5 mL), is likely micelles, while the retained zone is monomeric surfactant. In this experiment, the holdup volume is much greater than the column void volume because of the method of solute introduction. At 0.1% (v/v) surfactant concentration, almost 45 mg was pumped onto the column to reach a steady-state condition. At lower concentrations, of course, much longer times would be required to reach a steady state at the same flow rate. About 200 mg of the octylglucoside was added to reach saturation. The increase probably reflects the smaller molecular size of this surfactant. It is clear that caution must be exercised when surfactants containing mobile phases are employed to assure that the steady-state condition required for reproducible chromatography is reached.

When individual proteins were injected onto the reversed-phase column, their retention volumes fell into three broad classifications depending on the isocratic concentration of ethoxylated alcohol surfactant required for elution. Table I shows these groupings together with the molecular weights, isoelectric points, and average hydrophobicities of the proteins. Little correlation of retention with molecular weight is observed, although the most strongly retained solutes were of low molecular weight. The accessibility of these proteins to

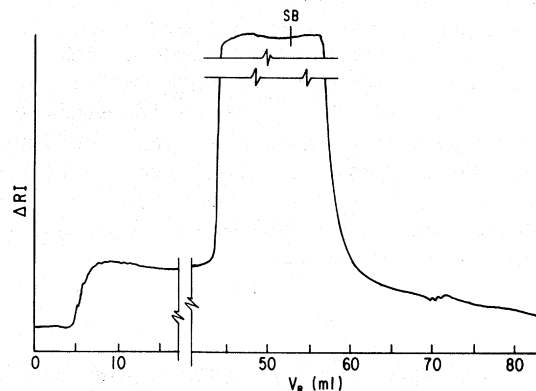


Figure 2. Surfactant frontalgram: mobile phase, 0.05 M phosphate (pH 7); flow rate, 1 mL min⁻¹; $R(OCH_2CH_2)_6OH$ concn = 0.1%; column, Supelcosil LC-8; mobile phase alone introduced at SB.

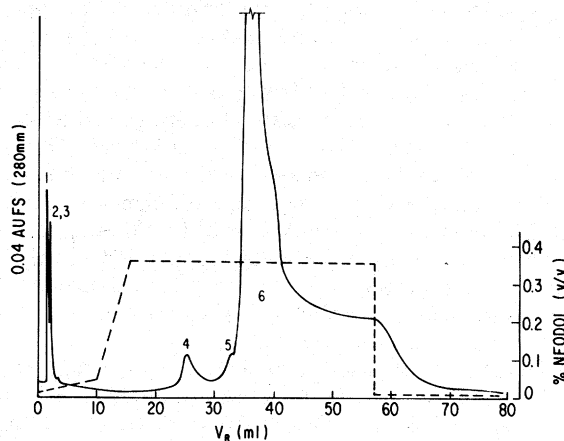


Figure 3. Gradient micellar chromatography of protein mixture: column, Supelcosil LC-8; mobile phase, $R(OCH_2CH_2)_6OH$ in 0.05 M phosphate (pH 7); (1) ovalbumin, (2) bovine serum albumin, (3) thyroglobulin, (4) chymotrypsinogen, (5) β -lactoglobulin, (6) lysozyme.

a larger percentage of the packing's surface area may account for the observed retention. Surface charge has minimal effect as indicated by the occurrence of basic proteins in all three classes. The average hydrophobicity has been correlated with protein properties such as solubility, aggregation phenomena, and thermal stability (13), but no direct or inverse correlation is observed here. Indexes of the protein's surface hydrophobicity might be more pertinent. This has been calculated (14), but for too few proteins to be useful in this study.

Unlike chromatography of low molecular weight solutes, where increases in concentration of surfactant in micelles produce similar magnitudes of reduction in retention volumes (2), small decreases of surfactant concentration caused, in the present experiments, exponential increases in protein retention. Therefore, to obtain separations of mixtures of selected proteins, shallow gradients were employed. Figure 3 illustrates

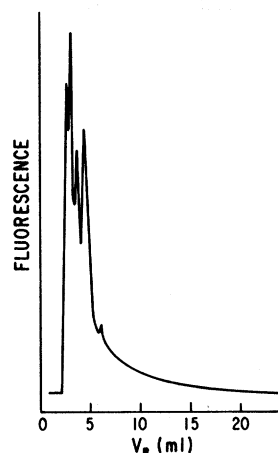


Figure 4. Micellar chromatography of commercial chymotrypsinogen A preparation: column, Supelcosil LC-8; mobile phase, 0.01% R-(OCH₂CH₂)₆OH in 0.05 M phosphate (pH 7).

the potential of micellar mobile phases for separating components with a wide range of properties, as is commonly the case for biological isolates. The last two peaks are partially obliterated by disturbances that are related to Schlieren and/or scattering effects in the detector induced by higher micelle concentrations. These are reproducible for a given gradient and can be compensated for with modern computer-assisted detectors and concomitant subtraction algorithms. Alternatively, on-line postcolumn derivatization techniques may be employed so that use of nonionic micellar mobile phases is not precluded by this disturbance.

For some purposes isocratic elution gives sufficient separation, although caution must be exercised to assure that no protein remains sorbed to the support. An example (Figure 4) demonstrates the utility of a micellar mobile phase to determine the homogeneity of a commercial protein preparation which was presumably of high purity. Since not all preparations produced complex chromatograms, it is presumed that some of the peaks are indicative of impurities but the presence of conformers was not ruled out.

A chromatogram of myoglobin is shown in Figure 5. Spectral evidence coupled with size exclusion chromatography provided information to aid in the identification of the first peak as myoglobin and to tentatively identify the second and later eluting components as apomyoglobin (protein with heme removed) and iron porphyrins, respectively.

Proteins were not eluted by solutions of octylglucoside at concentrations of 0.2 g/L (56 ergs/cm²) and 0.01 g/L (66 ergs/cm²). Micellar phases of this surfactant were, therefore, not tested. This information together with the above data permits conjecture with respect to possible retention mechanisms. A displacement model wherein the surfactant is viewed as a displacer is inappropriate because the necessary condition that it be the most strongly sorbed component (15) is not fulfilled. Here, although the surfactant is retained appreciably with buffer mobile phase, proteins are not observed to elute from the reversed-phase column. The surface tensions of most proteins in their natural conformations range from 66 to 71 ergs/cm² (10). One would expect, therefore, that both surfactants would effect elution at concentrations well below their critical micelle concentrations. Two rationales to reconcile this expectation with the experimental observations are proposed. One is that proteins undergo conformational changes upon sorption that reduce their surface tension, presumably by exposing more apolar amino acids to the surface. thus, further reduction in surface tension of the

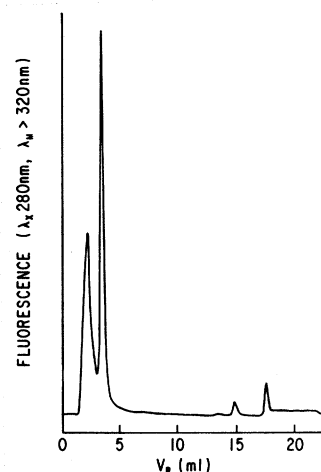


Figure 5. Monitoring beef heart myoglobin preparation. First peak is myoglobin, second peak is apomyoglobin (tentative), later peaks are iron porphyrins (tentative). Mobile phase was R-(OCH₂CH₂)₆OH in 0.05 M phosphate (pH 7).

mobile phase would be needed for desorption. the second proposal is that transfer of protein from the interface is facilitated by van der Waals attraction toward the micelle. In this study, the energetics for this mechanism appear reasonable, i.e., surface tension increases in the order stationary phase < solution < micelle < protein.

Some reports of the recovery from reversed-phase systems of substrates with biological activity have appeared (16) and the potential for micellar chromatography of proteins is clearly demonstrated. However, it remains for ongoing research to optimize the later separations and to ascertain whether they offer advantages for analyses over existing reversed-phase approaches with organic modifiers or over the use of high-performance ion-exchange techniques for rapid preparative isolations.

Registry No. β-Octylglucoside, 29836-26-8; chymotrypsinogen A, 9035-75-0.

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